Epstein-Barr virus latent membrane protein 1 induces CD69 expression through activation of nuclear factor-κB

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Abstract. Latent membrane protein-1 (LMP-1) of Epstein-Barr virus (EBV) promotes tumorigenesis. Here, we report that LMP-1 activates the immunoregulatory molecule CD69 gene transcription through a nuclear factor-κB (NF-κB)-dependent pathway. CD69 expression was upregulated in LMP-1-expressing EBV-immortalized human B-cell lines and an EBV-positive Burkitt's lymphoma cell line. LMP-1 expression increased CD69 expression at the transcriptional level. CD69 promoter was regulated by LMP-1 activation of NF-κB via the carboxy-terminal activation region 1 and 2. Promoter deletion analysis indicated that two NF-κB binding sites are necessary for activation of the CD69 promoter. Electrophoretic mobility shift analysis demonstrated that LMP-1 activates both NF-κB binding sites in the CD69 promoter. This is the first report of the regulation of CD69 expression by LMP-1, and this novel finding may, thus, represent an important link between the EBV oncoprotein LMP-1 and its critical role in the development of EBV-associated diseases.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous human γ-herpesvirus and was one of the first human virus linked to cancer (1). EBV can infect, transform and immortalize B-lymphocytes *in vitro*, giving rise to lymphoblastoid cell lines (LCLs), which display elevated levels of several cellular activation antigens (2). Consistent with this feature, persistent, latent EBV infection is present in several lymphoid malignancies including Burkitt's lymphoma (BL) (3).

EBV latent membrane protein-1 (LMP-1) is a key effector in EBV-mediated transformation of B cells (4). It consists of 386 amino acids and its carboxy-terminal cytoplasmic domain contains two carboxy-terminal activation regions (CTARs), CTAR-1 and CTAR-2. CTAR-1 and CTAR-2 are known to activate the nuclear factor-κB (NF-κB) signaling pathway (5-8). NF-κB is inactive in the cytosol because it is bound to IκBα and IκBβ, and becomes active after phosphorylation and subsequent degradation of IκBα and IκBβ (9). The released NF-κB from the complex translocates to the nucleus where it activates a variety of genes (9). The high-molecular weight complex, IκB kinase (IKK), which is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ, phosphorylates IκBs (9). Members of the p38 mitogen-activated protein kinase (MAPK) kinase kinase protein kinase family mediate the physiological activation of IKK (10). These kinases include NF-κB-inducing kinase (NIK) (11) and MAPK/extracellular signal-regulated kinase kinase 1 (12). CTAR-1 binds to tumor necrosis factor receptor-associated factors (TRAFs) (5), whereas CTAR-2 binds to the tumor necrosis factor receptor-associated death domain (TRADD) (6). NF-κB activation by the CTAR-1 and CTAR-2 domains of LMP-1 is likely to be mediated by the binding of TRAFs directly or indirectly to both the CTAR-1 and CTAR-2 domains (5-8). NF-κB activation by LMP-1 is mediated by NIK or a related MAPK kinase kinase, and subsequent activation of the IKK complex (7,13). NIK is activated by aggregated TRAF2 (13). Thus, binding of LMP-1 to TRAFs and TRADD initiates the formation of a signaling complex that leads to activation of NF-κB and MAPK (5-8), resulting in upregulation of several genes expression.

The human activation inducer molecule CD69 is a disulfide-linked transmembrane homodimeric glycoprotein (14). CD69 is not detected in peripheral blood lymphocytes, but it is expressed by small subsets of T and B cells in peripheral lymphoid tissues (15,16). CD69 acts as a signal transducer in inflammatory processes. Recent studies have established the role of CD69 as an intrinsic negative modulator of T cell responses (17). In the present report, we show that CD69 is a transcriptional target of LMP-1 activation of NF-κB via CTAR-1 and CTAR-2.

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Materials and methods

Cell lines. Raji is a human EBV-positive BL cell line, and LCL-Ao, LCL-Ka and LCL-Ku are EBV-immortalized human B-cell lines generated from peripheral blood mononuclear cells of healthy volunteers. These lymphoid cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10 or 20% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin.

Flow cytometry. The expression of CD69 was analyzed by flow cytometry with phycoerythrin (PE)-labeled mouse monoclonal antibody against CD69 (clone TP1.55.3; Beckman Coulter, Fullerton, CA). Analyses were carried out with isotype-matched control antibody. The cells were incubated with the antibody for 30 min, washed with cell WASH (Becton-Dickinson Immunocytometry Systems, San Jose, CA) and then subjected to analysis in the cytometer.

RNA detection. Total RNA was prepared from various cell cultures by TRIzol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 1 μg total cellular RNA using a PrimeScript RT-PCR kit (Takara Bio Inc., Otsu, Japan) with random primers. The primers used were 5'-CATAGCTCTCATTGCCTTA TCAGT-3' (forward) and 5'-CCTCTCTACCTGCGTATC GTTT-3' (reverse) for CD69, 5'-GTGACTGGACTGGAGG AGCC-3' (forward) and 5'-GAGGGAGTCATCGTGGT GGTG-3' (reverse) for LMP-1, and 5'-GTGGGGCGCCCCAG GCACCA-3' (forward) and 5'-CTCCTTAATGTCACGCACG ATTTC-3' (reverse) for β-actin. The length of the semiquantitative reverse transcription-PCR (RT-PCR) for each gene was: 30 cycles for CD69 and LMP-1, and 28 cycles for β-actin. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Plasmids and transfections. Luciferase assay was performed to confirm that LMP-1 induces the CD69 promoter activation. Approximately $3x10^5$ 293T cells per plate were transfected by the calcium phosphate DNA co-precipitation method. All transfections included appropriate reporter and effector plasmids. The expression plasmids pSG5-LMP-1, pSG5-LMP-1Δ187-351, pSG5-LMP-1Δ349 and pSG5-LMP-1Δ194-386 were kindly provided by Dr Martin Rowe (University of Wales College of Medicine, Cardiff, UK) (18,19). The CD69 promoter-luciferase gene constructs have already been described (20,21). The single and combined internal deletion mutants of NF-κB sites were constructed by deletion of the NF-κB sites of the plasmid pAIM 255-LUC. The IκB$\alpha\Delta$N- and IκB$\beta\Delta$N-dominant negative mutants are IκBα and IκBβ deletion mutants lacking the amino-terminal 36 and 23 amino acids, respectively (22,23). The dominant negative mutants of IKKα, IKKα (K44M), IKKβ, IKKβ (K44A), IKKγ, IKKγ (1-305) and NIK, NIK (KK429/430AA) have been described previously (24,25). Plasmids for truncated TRAF2 and TRAF5 proteins retaining only the TRAF domain, ΔTRAF2 and ΔTRAF5, have been described previ-

ously (26,27). In all cases, the reference plasmid phRL-TK, which contains the *Renilla* luciferase gene under the control of the herpes simplex virus thymidine kinase promoter, was cotransfected to correct for transfection efficiency. After 24 h, the transfected cells were collected by centrifugation, washed with phosphate-buffered saline and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase assays were conducted using the dual luciferase reporter system (Promega), in which the relative luciferase activity was calculated by normalizing transfection efficiency according to the *Renilla* luciferase activities.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA). Nuclear proteins were extracted as described by Antalis and Godbolt (28) with some modifications, and NF-κB binding activity to the NF-κB element was examined by EMSA. Briefly, 5 μg of nuclear extracts were preincubated in a binding buffer containing 1 μg polydeoxyinosinic-deoxycytidylic acid (GE Healthcare Biosciences, Buckinghamshire, UK), followed by the addition of ^{32}P-labeled oligonucleotide probes containing the NF-κB element. The mixtures were incubated for 15 min at room temperature. The DNA protein complexes were separated on 4% polyacrylamide gels and visualized by autoradiography. The probes and competitors used were prepared by annealing the sense and antisense synthetic oligonucleotides as follows: the NF-κB element (κB1) of the CD69 gene (5'-GATCC AGACAACA<u>GGGAAAACCC</u>ATACTTC-3'); the NF-κB element (κB2) of the CD69 gene (5'-GATCCAGAGTCT<u>GGG AAAATCCC</u>ACTTTCC-3'); a typical NF-κB element from the IL-2 receptor α chain (IL-2Rα) gene (5'-GATCCGGCAG <u>GGGAATCTCCC</u>CTCTC-3') and an AP-1 element of the IL-8 gene (5'-GATCGTGA<u>TGACTCA</u>GGTT-3'). The above underlined sequences represent the NF-κB and AP-1 binding sites, respectively.

Results

Upregulated CD69 expression in EBV-immortalized human B-cell lines and EBV-positive BL cell line. We examined first whether CD69 upregulation is a general feature of EBV-infected B cells. All EBV-immortalized B-cell lines, LCL-Ao, LCL-Ka and LCL-Ku, and EBV-positive BL cell line, Raji, constitutively expressed CD69 on the cell surface (Fig. 1A). In contrast, CD69 was hardly expressed on normal peripheral blood mononuclear cells (data not shown).

LMP-1 upregulates CD69 mRNA and protein expression. EBV-immortalized B-cell lines and Raji cells constitutively express LMP-1 (29,30). Therefore, we analyzed the induction of CD69 expression by LMP-1. Transient expression assays using a mammalian expression vector for LMP-1 were performed in 293T cells. In 293T cells transfected with empty vector (pSG5), CD69 mRNA was not detectable as determined by RT-PCR analysis (Fig. 1B). In contrast, ectopic expression of LMP-1 induced the expression of CD69 mRNA in 293T cells. Next, we investigated whether LMP-1 also transcribed the endogenous CD69 gene. The expression of CD69 antigen on the cell surface of 293T cells increased after ectopic expression of LMP-1 in 293T cells, and such increase was time-dependent (Fig. 1C).
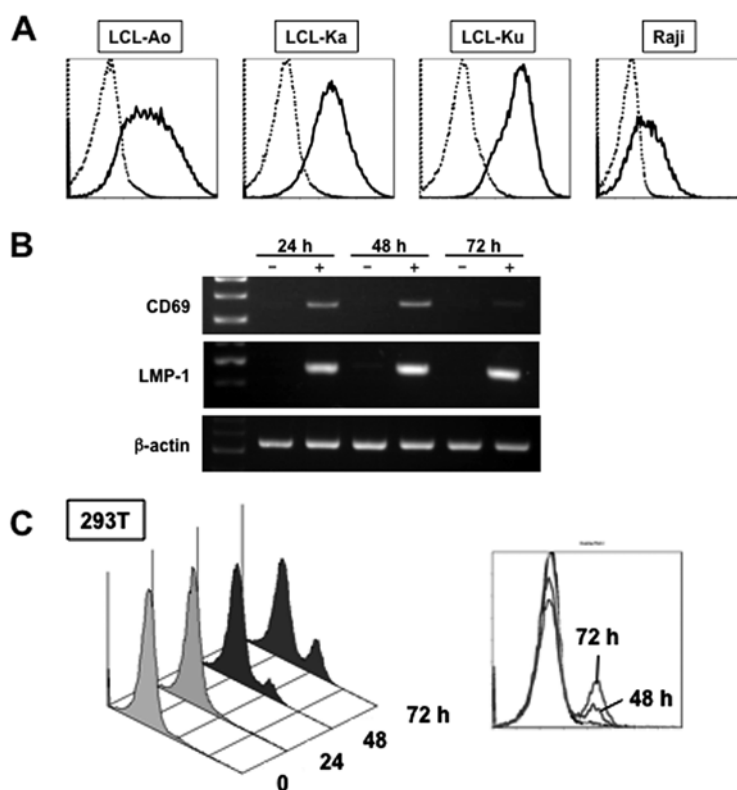
Figure 1. LMP-1 induces CD69 expression. (A) Expression of CD69 on the cell surface of EBV-infected B cells. Flow cytometry of the indicated cell lines. Cells were either stained with an anti-CD69-PE antibody or an isotype control-PE antibody, and subjected to flow cytometry. (B) Ectopic expression of LMP-1 in 293T cells upregulates CD69 mRNA expression. 293T cells were transfected with plasmid encoding LMP-1 or empty vector. Total RNA was isolated from 293T cells at the indicated times, and RT-PCR was carried out for CD69, LMP-1 and β-actin (loading control). (C) Endogenous CD69 antigen expression on 293T cells after ectopic expression of LMP-1 for 24, 48 and 72 h. The right panel shows an overlay histogram of flow cytometry data.

LMP-1 activates CD69 promoter. To determine whether LMP-1 regulates CD69 promoter activity, transient expression assays using the reporter plasmid pAIM 1.4-LUC and either an expression vector for LMP-1 or empty vector (pSG5) were performed in 293T cells. LMP-1 induced relative levels of CD69 promoter-directed luciferase expression in a dose-dependent manner, suggesting that LMP-1 functionally activates CD69 promoter (Fig. 2A). To map the region in the LMP-1 protein that mediates activation of CD69 promoter, LMP-1 mutants were expressed in 293T cells and their effect on CD69 promoter activity was investigated. The LMP-1 mutants used included LMP-1Δ187-351 (which contains only CTAR-2 in the carboxy-terminus), LMP-1Δ349 (which lacks CTAR-2), and LMP-1Δ194-386 (in which the entire carboxy-terminal cytoplasmic region is deleted) (Fig. 2B, left panel). In cells that expressed LMP-1Δ194-386, CD69 promoter activity was not significantly increased (Fig. 2B, right panel). In contrast, LMP-1Δ187-351 induced 50% of wild-type LMP-1 CD69 promoter activation. Furthermore, LMP-1Δ349 showed substantial impairment of CD69 promoter activation. These results suggest that LMP-1 activates CD69 expression via the cooperative activity of CTAR-1 and CTAR-2 signaling motifs.

LMP-1 activates the CD69 promoter via the NF-κB signaling pathway. CTAR-1 and CTAR-2 are known to activate the NF-κB signaling pathway (5-8). Based on the discussed background, we tested the ability of the dominant interfering mutants of IκBα,

IκBβ, IKKγ, TRF2 and TRAF5, and kinase-deficient mutants of IKKα, IKKβ and NIK to inhibit LMP-1-mediated transactivation of CD69-driven reporter gene activity. Expression of each of these inhibitory mutants inhibited LMP-1-induced activation of CD69 promoter (Fig. 2C). These results demonstrated that the signaling components, TRAFs, NIK and IKKs, which are involved in the activation of NF-κB, are also necessary for LMP-1 transactivation of the CD69 promoter.

LMP-1 activates the CD69 promoter through two NF-κB sites. The CD69 promoter contains three putative binding sites for NF-κB [positions -373 (κB3), -223 (κB2) and -160 (κB1)], a putative binding site for early growth response (EGR) at -69, a composite binding site for Sp1 and EGR at -56, a cyclic adenosine 3',5'-monophosphate response element-binding protein (CREB) binding site at -46, and two putative AP-1 binding sites at -16 and +1 (Fig. 3A) (20). To study the role of NF-κB sites in the transcriptional induction of CD69 gene by LMP-1, we transfected 293T cells with several 5'-deletion fragments extending from position -1.4 k to -78 (Fig. 3A), and an expression vector for LMP-1. Progressive removal of 5'-sequence up to position -255 did not significantly inhibit the induced promoter activity, suggesting that the 271-bp fragment, spanning positions -255 to +16, contained the LMP-1-responsive elements (Fig. 3A). Further deletion of upstream sequences up to position -170 resulted in significant loss of responsiveness to LMP-1. Additional removal of the
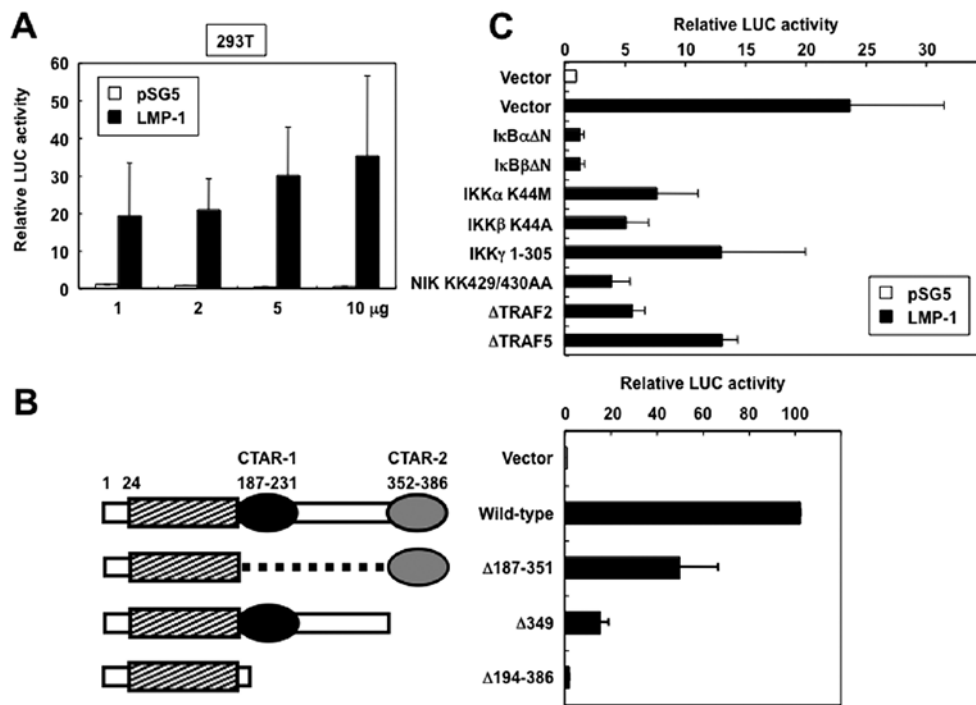
Figure 2. LMP-1 activates the CD69 promoter via the NF-κB signaling pathway. (A) LMP-1 activates the CD69 promoter. 293T cells were transfected with pAIM 1.4-LUC (0.2 ng) together with increasing amounts (1-10 μg) of pSG5 or pSG5-LMP-1. Cells were harvested 24 h post-transfection and luciferase activity was measured with a luminometer. The activities are expressed relative to that of cells transfected with pAIM 1.4-LUC and pSG5, which was defined as 1. (B) Deletions in CTAR-1 and CTAR-2 abrogate the effect of LMP-1 on CD69 promoter activity. 293T cells were cotransfected with plasmids encoding wild-type LMP-1 or various mutants of LMP-1 (5 μg) (left) and with pAIM 1.4-LUC (20 ng). The results are represented as fold induction by wild-type or mutant LMP-1 relative to the vector alone. (C) Effects of dominant negative mutants on LMP-1-induced CD69 promoter activation in 293T cells. pSG5 or pSG5-LMP-1 (2 μg) was cotransfected with pAIM 1.4-LUC (0.2 ng) and the indicated dominant negative mutants or empty vector (2 μg). The activities are expressed relative to that of cells transfected with pAIM 1.4-LUC and empty vectors, which was defined as 1. Data are mean ± SD of three experiments. LUC, luciferase.
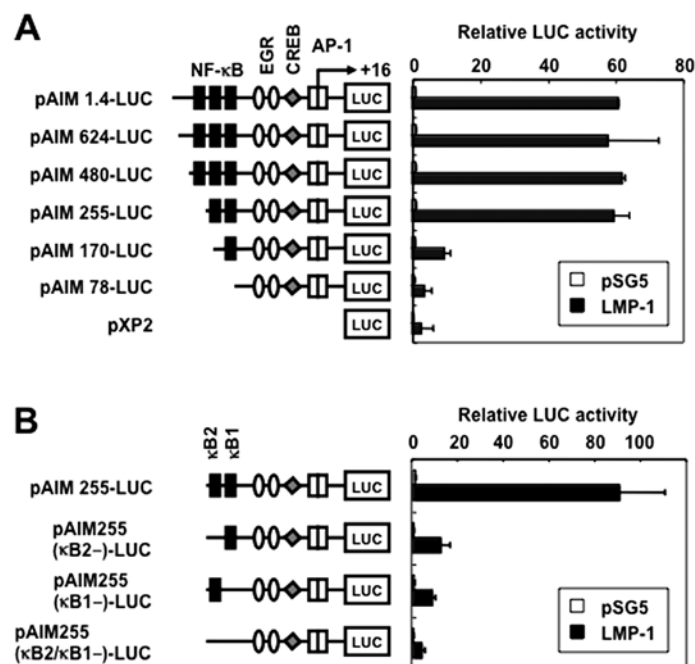

Figure 3. Two NF-κB sites of the CD69 gene promoter are required for LMP-1-induced CD69 gene transcription. (A) 293T cells were transfected with the indicated CD69 luciferase reporter constructs (0.2 ng) and either empty vector or pSG5-LMP-1 (5 μg). The deleted plasmids are indicated on the left side of the figure. The activities are expressed relative to that of cells transfected with pXP2 and an empty vector, which was defined as 1. (B) 293T cells were transfected with pAIM 255-LUC, pAIM 255-LUC with deleted distal NF-κB site [pAIM 255 (κB2-)-LCU], pAIM 255-LUC with deleted proximal NF-κB site [pAIM 255 (κB1-)-LUC] or pAIM 255-LUC with double deleted [pAIM 255 (κB2/κB1-)-LUC] (0.2 μg) together with either empty vector or pSG5-LMP-1 (5 μg). Schematic diagrams of the CD69 reporter constructs containing the wild-type (pAIM 255-LUC) and internal deletion mutants of κB1 and/or κB2 motifs are indicated on the left side of the figure. The activities are expressed relative to that of cells transfected with pAIM 255-LUC and an empty vector, which was defined as 1. Data are mean ± SD of three experiments. LUC, luciferase.
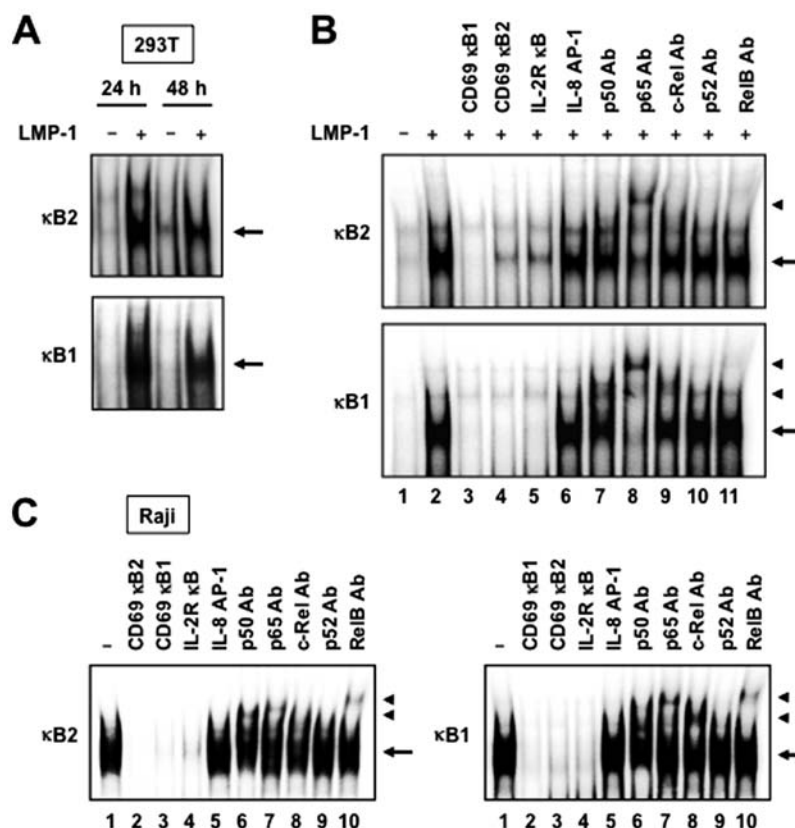
Figure 4. LMP-1 induces NF-κB binding to DNA. (A) 293T cells were transfected with control plasmid or LMP-1 expression plasmid. Nuclear proteins were extracted at the indicated times. The binding of NF-κB to DNA was measured by EMSA experiments. (B and C) Nuclear extracts from 293T cells transfected with (B) control plasmid or LMP-1 expression plasmid or (C) Raji cells were incubated with the labeled double-stranded DNA probes representing the CD69 κB1 and κB2 sites. Nuclear extracts were subjected to competition analysis with an excess of unlabeled double-stranded oligonucleotides representing the CD69 κB1 and κB2 sites (lanes 3 and 4 in B and lanes 2 and 3 in C, respectively), a consensus NF-κB site from the IL-2Rα promoter (lane 5 in B and lane 4 in C) or an AP-1 site from the IL-8 promoter (lane 6 in B and lane 5 in C). The indicated unlabeled oligonucleotides were incubated with nuclear extracts for 15 min prior to binding reactions. Nuclear extracts were also subjected to supershift assays with either no antibody (lane 2 in B and lane 1 in C) or the indicated antibodies (Ab) (lanes 7-11 in B and lanes 6-10 in C). The antibodies were incubated with nuclear extracts for 45 min prior to binding reactions. Arrows, specific complexes; arrowhead, the DNA binding complex supershifted by the antibody. Representative results of three experiments of similar results.

5'-sequences up to position -78 further affected the inducible promoter activity.

Two potential NF-κB-binding sequences were identified at positions -160 (κB1) and -223 (κB2) spanning positions -255 to +16 bp. κB1 and κB2 were identical to those found in the gene promoters of c-myc and IL-6, respectively (31). To test the relative contribution of the NF-κB binding sites to the LMP-1-mediated activation of CD69, plasmids with internal deletion mutants of these sites in the CD69 promoter were transfected (Fig. 3B). Single deletion of the κB1 or κB2 site resulted in reduction of the inducible activity. Double deletions of the κB1 and κB2 sites further reduced Tax-mediated activation of this reporter construct. These results indicate that the two NF-κB binding sites in the CD69 promoter regulate LMP-1-induced upregulation of CD69.

LMP-1 induces NF-κB binding to DNA. Since both NF-κB binding sites of the CD69 promoter are required for LMP-1-mediated CD69 gene transcription, we studied the ability of LMP-1 to activate NF-κB binding to DNA. For this purpose, 293T cells were transfected with control plasmid (empty vector) or LMP-1 expression plasmid for 24 or 48 h, followed by extraction of nuclear proteins. EMSA demonstrated that LMP-1

increased the binding of NF-κB to ^{32}P-labeled oligonucleotides representing the κB1 and κB2 sites (Fig. 4A). The specificity of DNA-protein complex formation was determined by competition studies with unlabeled competitors. As expected, excess of cold CD69 κB1 or κB2 double-stranded oligonucleotide, or consensus NF-κB site from the IL-2Rα promoter, effectively competed with the labeled probes and eliminated the binding of nuclear extracts from 293T cells transfected with LMP-1 expression plasmid (Fig. 4B, lanes 2-5). In contrast, the unlabeled IL-8 AP-1 element did not compete with labeled probes (Fig. 4B, lanes 2 and 6). The nucleoproteins binding to both NF-κB sites were composed of p50, p65 and c-Rel subunits of the NF-κB family, as demonstrated by supershift experiments with specific antibodies (Fig. 4B, lanes 7-9).

Finally, to determine the role of LMP-1 on endogenous NF-κB binding to DNA, we measured NF-κB binding to respective NF-κB elements in the CD69 promoter in LMP-1- and CD69-expressing Raji cells. As expected, protein complexes bound to both κB1 and κB2 sites were detected in nuclear extracts from Raji cells (Fig. 4C, lane 1). The specificity of DNA-protein complexes in these extracts was determined by competition studies using unlabeled competitors. As observed in nuclear extracts from 293T cells transfected with LMP-1

expression plasmid, cold κB1 and κB2 oligonucleotides, and consensus NF-κB site from the IL-2Rα promoter, but not the IL-8 AP-1 element, efficiently competed with labeled probes (Fig. 4C, lanes 1-5). Antibodies against p50, p65, c-Rel and RelB induced a supershift of the DNA-protein complexes (Fig. 4C, lanes 6-8 and 10). Taken together, the results indicate that NF-κB proteins bind to both κB elements of the CD69 promoter in LMP-1- and CD69-expressing Raji cells.

Discussion

The main issue addressed in this study was whether LMP-1 induces CD69 gene transcription and the possible mechanisms underlying this activity. In this study, we showed that EBV LMP-1 activates CD69 gene transcription through an NF-κB-dependent pathway. The activation of CD69 promoter was mediated cooperatively by CTAR-1 and CTAR-2 of LMP-1, as demonstrated by ectopic expression of LMP-1 mutants. Interestingly, previous studies have demonstrated that CTAR-1 and CTAR-2 cooperatively activate the NF-κB signaling pathway (5-8), and that CD69 promoter harbors three κB consensus sites (κB1-κB3) (20). The results also showed that both κB1 and κB2 domains of the CD69 promoter were absolutely necessary for LMP-1-induced transcription.

The present results indicate that LMP-1 induces CD69 promoter activation through NIK-, IKKα- and IKKβ kinase-dependent pathways. Dominant negative forms of NIK, IKKα, IKKβ- and IKKγ inhibited LMP-1-induced CD69 promoter activation. These results are consistent with previous evidence that NIK and IKKs are implicated in the pathways through which LMP-1 induces NF-κB activation (7,13). LMP-1 CTAR-1 directly recruits TRAF1, 2, 3 and 5 whereas CTAR-2 indirectly recruits TRAF2 and 6 (5-8). CTAR-1 activates TRAF2-, NIK- and IKKα-mediated NF-κB pathways, and CTAR-2 activates TRAF6-, IKKα-, IKKβ- and IKKγ-mediated NF-κB pathways (7). Overexpression of dominant negative forms of TRAF2 and TRAF5 inhibited LMP-1-mediated CD69 promoter activation, indicating that NF-κB is an important component of LMP-1-mediated CD69 gene induction from TRAFs-interacting sites.

CD69 has been found to be rapidly upregulated on all the leukocyte lineages studied, upon activation with the corresponding stimuli (14). CD69 expression has also been reported in infections (32-35). Earlier studies showed that CD69 regulates the immune response by modulating the expression of various cytokines. CD69-deficient mice show increased anti-tumor and autoimmune responses, which are caused at least in part by increased production of proinflammatory cytokines and chemokines (36,37). More recently, tumor-derived CD69⁺ T cells have been found to induce immune tolerance in the tumor environment (38). CD69 is reported to be a critical negative regulator of immune activation during intracellular bacterial infection (35). LMP-1-mediated CD69 may have an important role in the immune surveillance evasion by EBV-infected cells.

In summary, our experiments indicate that LMP-1 CTAR-1 and -2 can regulate CD69 gene at the transcriptional level via two NF-κB-binding sites in its promoter, and TRAFs, NIK and IKKs are effectors of CD69 promoter activation from LMP-1. Further studies are necessary to resolve the question of the role of CD69 in EBV-associated diseases.

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